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Airway cell patterns in patients suffering from COPD and OSAS (Overlap Syndrome)

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Summary
Background: Obstructive sleep apnea syndrome (OSAS) and chronic obstructive pulmonary disease (COPD) are two diseases that often coexist within an individual. This coexistence is known as Overlap Syndrome (OS). Both diseases are characterized by local and systemic inflammations, but no studies to date have investigated local airway inflammation in patients suffering from Overlap Syndrome.
Methods: We performed a Berlin Questionnaire to evaluate the presence of the principal OSAS symptoms, a pulmonary function test, and then a nocturnal oximetry and polysomnography in 72 patients that were divided into five groups: OS ($n = 18$), COPD ($n = 15$), OSAS ($n = 16$), 12 obese without OSAS or COPD, and one control group of 11 normal subjects. All patients underwent sputum induction and the analysis of cell patterns were evaluated in all groups. The relationship with the degree of obesity, airway obstruction and OSAS severity was also evaluated.
Results: The percentage of neutrophils in induced sputum was higher in OS (74.33% ± 14.8), COPD (63.33% ± 13.22) and OSAS (60.69% ± 17.6) subjects compared with control groups of obese (43.5% ± 17.49) and normal weight (32.04% ± 12.26). No difference was found among Overlap, COPD, and OSAS patients ($p = 0.56$). A negative correlation was found between PaO_2 and percentage of airway neutrophils ($r = -0.29$, $p < 0.05$); similarly, no correlations arose between BMI, FEV_1 or ODI.

Abbreviations: COPD, chronic obstructive pulmonary disease; OSAS, obstructive sleep apnea syndrome; OS, Overlap Syndrome; IS, induced sputum; RDI, respiratory disturbance index; ODI, oxygen desaturation index; BMI, body mass index; TST90, total sleep time with SaO_2 under 90%.

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Conclusion: Patients suffering from Overlap Syndrome present a high percentage of neutrophils in induced sputum like patients affected by COPD or OSAS alone. Our result suggests that airway inflammations is always involved in all of these diseases, even though probably sustained by different mechanisms.

Introduction

Chronic obstructive pulmonary disease (COPD) is a frequent pulmonary pathology whose prevalence has been estimated to be 1% of the general population and up to 5—10% of the older adult population; it is a condition of progressive deterioration of the respiratory system characterized by an obstruction of the pulmonary airways and decreasing expiratory airflow. COPD is defined by the presence of an obstructive ventilatory defect, characterized by a FEV_1/FVC ratio less than 70%[1] and it is associated with an abnormal inflammatory response of the lungs to noxious particles or gases, primarily caused by cigarette smoking.[2]

Obstructive sleep apnea syndrome (OSAS), a highly prevalent disorder affecting approximately 4% of the adult population,[3] is characterized by recurrent upper airway collapse and episodes of hypoxia/reoxygenation during sleep.[4]

OSAS sometimes coexists with COPD and so it creates a disease with particular characteristics commonly known as "Overlap Syndrome."(OS).[5] OS can be expected to be present in 0.5—1% of the population over 40 years of age.[6] The prevalence of OSAS is the same in subjects with COPD as those without COPD, the association between COPD and OSAS occurring by chance. OS predisposes to daytime hypercapnia and hypoxemia independently of lung function.[7] Nocturnal arterial oxyhemoglobin desaturation and diurnal hypoxemia and hypercapnia are more pronounced in patients with coexistent COPD and OSA than in patients with either condition. Patients with both conditions have a greater risk of pulmonary hypertension and heart failure compared to patients with COPD or OSA alone.[8,9]

Both diseases are characterized by local and systemic inflammations. Several studies found an increase of local biomarkers of inflammations in OSAS: Rubinstein[10] showed an increase in the percentage of PMN (Polymorphonuclear leukocyte) in the nasal mucosa of patients suffering from OSAS, while our group found an increase of the neutrophilic percentage in induced sputum.[11]

On the other hand, in COPD also, much of the evidence demonstrates that airway inflammation is central to the pathogenesis of both airway remodelling and parenchymal destruction.[12] The cellular inflammatory response is characterized by an increase in neutrophils, and CD8-positive T-lymphocytes in small and large airways as well as in lung parenchyma.[13]

However, as far as we know, no studies to date have investigated airway inflammation characteristics in patients with both diseases. We investigated the hypothesis according to which the presence of OSAS and COPD can increase local inflammation and pulmonary damage.

In order to test this hypothesis, we investigated the bronchial inflammatory cell profile in OSAS, COPD and OS patients by using the analysis of the induced sputum which — even if it does not cover all the inflammatory and structural changes of the lungs — it can be useful in evaluating the cell airway composition in pulmonary disease.

Material and methods

Subjects

160 subjects afferent to Sleep Laboratories of the Institute of Respiratory Diseases, University of Foggia and Fondazione Maugeri, Cassano Murge (Bari) with suspected OSAS were initially screened. 88 subjects respected inclusion criteria (no history of systemic diseases, no current smokers or ex-smokers for at least 2 years, FEV_1 >30% of predicted, free from inhaled steroids or other anti-inflammatory drugs for at least 6 months, no COPD exacerbations in the previous year), 7 subjects refused their consent to the study; furthermore, 20 patients were excluded because the quality of induced sputum was not suitable for analysis, with the result that 61 subjects completed the study. Indeed, 11 lean, healthy volunteers were also enrolled for control group purposes.

None of the subjects was using CPAP therapy at that time. The Berlin Questionnaire[14] used for screening the presence of OSAS symptoms, a pulmonary function test, a nocturnal oximetry and induced sputum test were performed in all subjects. All healthy and COPD subjects who presented no risk of OSAS at the Berlin Questionnaire and no alterations of nocturnal oximetry, did not undergo a polysomnography. Subjects with a high risk of OSAS in the Berlin questionnaire, ODI >10 or BMI>25 kg/m^2, nevertheless performed a full polysomnography. All subjects written informed consent, and the study was approved by the institutional ethics committee. A complete physical examination was performed, including neurological, cardiopulmonary, ear, nose and throat examinations.

According to the result of the pulmonary functions test and polysomnography data, we divided the patients into 5 groups: group 1 (COPD/OSAS +/+) 18 subjects, group 2 (COPD/OSAS +/−) 15 subjects, group 3 (COPD/OSAS −/+) 16 subjects, group 4 (Obese COPD/OSAS −/−) 12 subjects and group 5 (lean COPD/OSAS −/−) 11 subjects.

Nocturnal oximetry

Nocturnal haemoglobin oxygen saturation was evaluated by finger pulse oximetry (Minolta, Pulsox DP 8, Japan). A pattern of oximetry tracing positive for OSAS was defined as a cyclical oxygen desaturation and a drop in oxygen saturation >4%. The test was considered valid if at least 6 h recording were analyzable.

Pulmonary function testing

Pulmonary function tests (Vmax 6200 Autobox, SensorMedics, Yorba Linda, CA) were performed before starting the study, in order to exclude an obstructive lung disease defined as a FEV1 % predicted less than 80 and a FEV_1/FVC ratio less than 0.7.

Polysomnography

Overnight full polysomnography was performed in the sleep laboratory (Sleep Lab 1000p, Aequitron Medical, MN, USA). Sleep-disordered breathing was quantified by RDI (respiratory disturbance index), the average number of respiratory events (apneas + hypopneas) per hour of sleep. This was calculated by dividing the total number of respiratory events during the study by the number of hours of sleep. Sleep-disordered breathing was quantified according to standard criteria.[15]

Induced sputum technique

Inhalation procedure: after baseline FEV_1 and FVC measurements, salbutamol was given to subjects by inhalation (200 mg by MDI, metered-dose inhaler) and subjects inhaled hypertonic (4.5%) saline nebulized for three periods of 5 min each. An ultrasonic nebulizer (De Vilbiss 65, De Vilbiss Corporation, Somerset, PA, USA), nebulized saline solutions.

Sputum processing: the collected sputum samples were examined within 2 h. Selected portions of the sputum sample originating from the lower respiratory tract were analyzed. Dithiothreitol (DTT, Sputolysin, Calbiochem Corp, San Diego, CA, USA), freshly prepared in a dilution of one in 10 with distilled water, was added to a volume equal to 4 times the weight of the sputum portion. Selected sputum was placed in a shaking water-bath at 37 °C for 20 min and homogenized. It was further diluted with phosphate-buffered saline in a volume equal to the sputum plus DTT. The suspension was filtered through gauze to remove any mucus and was centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in a volume of PBS equal to that of the sputum plus DTT and PBS, as above. Total cell count (TCC) and viability (Trypan blue exclusion method) were determined using a Burkers chamber haemocytometer. The cell suspension was placed in a Shandon 3 cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA) and cytospins were prepared at 450 rpm for 6 min. Cytospin slides were fixed by methanol and stained by May Grunwald Giemsa for an overall differential cell count on 500 nucleated non-squamous cells. Only samples with cell viability >50% and squamous cell contamination <20% were considered adequate. The slides were read by two independent investigators.

Statistical analysis

Differences among five groups (Overlap, COPD, OSAS, Obese, normal) were tested by the ANOVA for normally distributed variables and the Kruskal–Wallis test for non-normally distributed variables. *Post hoc* analyses by Bonferroni correction were performed. Correlations between functional and biological parameters, and inflammatory cells were evaluated by means of Spearman's rank correlation test. Significance was defined as a p value less than 0.05. Data are presented as mean ± Standard Deviation.

Results

There were no significant differences in BMI between Overlap, OSAS and Obese patients ($p = 0.29$); however, it was significant among all groups ($p < 0.001$). This was related to a lower BMI in COPD and the control lean group. Although there was no RDI difference among Overlap and OSAS patients ($p = 0.17$), by design, there was a difference with control group ($p < 0.001$). There was no difference in pulmonary function between overlap and COPD; Table 1 shows the characteristics of the five groups.

The percentage of neutrophils (Fig. 1) in induced sputum was higher in Overlap, COPD and OSAS group, compared with obese and lean subjects without OSAS or COPD. No significant difference was found among the three groups of patients: overlap vs group COPD (74.33 ± 14.8 vs 63.33 ± 13.22, $p = 0.37$); overlap vs OSAS (74.33 ± 14.8 vs 60.69 ± 17.6, $p = 0.15$); and between COPD and OSAS (63.33 ± 13.22 vs 60.69 ± 17.6, $p = 0.99$). The percentage of macrophage cells was lower in Overlap, COPD and in OSAS patients than control groups ($p < 0.05$). There was no significant difference among these three groups. In Overlap patients the percentage of lymphocytes (0.06 ± 0.24) was lower than all other groups ($p < 0.01$). Finally, the eosinophils were higher in COPD than the other groups ($p < 0.05$).

Concerning gas exchange, no significant difference was shown in $PaCO_2$ and PaO_2 values among three groups of patients. There was no difference in TST90 between overlap and OSAS group ($p = 0.39$).

A negative correlation was found between diurnal PaO_2 and percentage of neutrophils in airway ($r = -0.29$, $p < 0.05$), no correlations were forthcoming with BMI ($r = 0.07$; $p = 0.61$), FEV_1($r = -0.21$; $p = 0.16$) RDI or ODI ($r = -0.01$; $p = 0.93$) and $PaCO_2$ (Fig. 2) and with TST90 ($r = 0.24$; $p = 0.16$).

Discussion

The aim of this study was to evaluate airway inflammation in Overlap Syndrome and establish if there were some differences with respect to OSAS and COPD. Our data demonstrates that patients suffering from Overlap Syndrome show a higher percentage of neutrophils in their airway than lean and obese subjects without COPD or OSAS, but no difference with patients suffering from COPD or OSAS alone. We can hypothesize that different mechanisms are at the basis of the increased airway inflammations in COPD and OSAS, so that when they are both present, like in the Overlap Syndrome, each of them contributes to the development of a pulmonary inflammation.

The first data that rose from this study is the increase of eosinophils percentage in the induced sputum of COPD patients. These results were also observed in patients with

Table 1 Characteristics of groups and airway cells compositions in each of them.

n	Overlap (1)	COPD (2)	OSAS (3)	Obese no (4)	Lean no (5)	*p*
	18	15	16	12	11	
Age (year)	64.93 ± 10.25[a]	66.13 ± 11.94[a]	58.5 ± 7.16[a]	46.42 ± 9.96	49.55 ± 7.92	<0.01
BMI (kg/m^2)	35.29 ± 6.56[b]	30.58 ± 3.9[b]	38.62 ± 9.4[b]	34.29 ± 7.17	20.41 ± 2.12	<0.01
RDI (events/h)	38.85 ± 17.28[a]		43.42 ± 26.42[a]	5.35 ± 2.78		<0.001
ODI (events/h)	38.89 ± 16.11[a]	3.13 ± 2.26	43.56 ± 27.13[a]	3.51 ± 3.16	0.64 ± 0.81	<0.001
TST90 (%)	49.42 ± 36.29[a]	1.1 ± 1.28	36.38 ± 27.4[a]	0.92 ± 1.08	0	<0.001
FEV_1 (%)	56 ± 14.28[a]	59.69 ± 14.01[a]	94.31 ± 13.56	92.75 ± 18.13	111.09 ± 18.34	<0.001
FVC (%)	80.47 ± 11.9[a]	74.92 ± 16.18[a]	94.44 ± 13.86	93.25 ± 19.03	113 ± 18.65	<0.001
FEV_1/FVC	0.53 ± 0.1[a]	0.62 ± 0.07[a]	0.79 ± 0.06	0.87 ± 0.12	0.90 ± 0.09	<0.001
PaO_2 (mmHg)	65.36 ± 7.99[a]	73.64 ± 14.92[a]	72.09 ± 9.83[a]	86.23 ± 13.19		<0.01
$PaCO_2$ (mmHg)	44.87 ± 5.82	40.65 ± 6.67	43.39 ± 4.95	40.48 ± 3.21		0.09
Macrophages (%)	23.94 ± 13.63[a]	27.6 ± 15.38[a]	35.31 ± 15.8[a]	54.42 ± 18.7	64.91 ± 12.77	<0.01
Neutrophils (%)	74.33 ± 14.8[a]	63.33 ± 13.22[a]	60.69 ± 17.06[a]	43.5 ± 17.49	32.04 ± 12.26	<0.01
Eosinophils (%)	1.28 ± 1.96	6.07 ± 8.18	0.59 ± 0.95	1.33 ± 1.56	0.09 ± 0.21	<0.001
Lymphocytes (%)	0.06 ± 0.24	4.89 ± 3.02	2.03 ± 3.21	0.67 ± 1.04	1.04 ± 1.27	<0.01

NO: Non OSAS. Data are expressed as mean ± SD.
[a] Significant difference vs normal weight and/or obese.
[b] Significant difference vs normal weight but not vs obese.

stable COPD, but its relationship to airflow limitation is controversial.[24] It has been argued that sputum eosinophilia is related to concomitant features of asthma.[25] This link would indicate that the pathophysiologic entities underlying the clinical phenotypes in COPD may be diverse and are still largely unknown.

The reductions of lymphocytes in OS are probably only a consequence of an increase in the percentage of neutrophils.

What is perhaps more interesting is our observation that in Overlap Syndrome there is a high percentage of neutrophils in IS, and this increase of neutrophils is the same as we found in COPD ad OSAS patients.

Neutrophils are front-line defensive cells of the immune system and a source of reactive oxygen metabolites, inflammatory cytokines, lipid mediators, antibacterial peptides, and tissue-damaging enzymes.[16] The in-vivo evidence linking neutrophils with COPD is abundant.

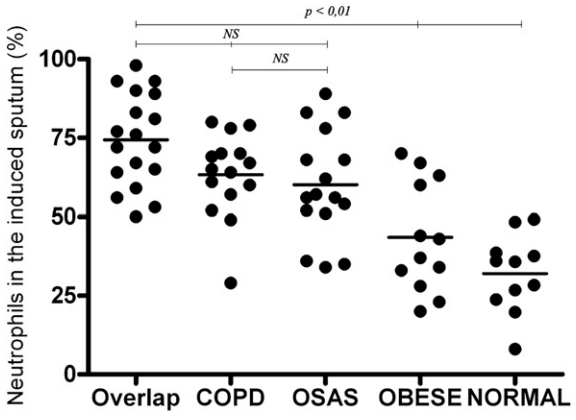

Figure 1 Percentage of neutrophils in the five groups: higher in OS, COPD and OSAS than in control groups, any difference inside three groups of patients.

Analyses of induced sputum[17,18] and airway lavage fluid[19] consistently demonstrate increased neutrophil counts and neutrophil-derived enzyme levels in COPD both when stable and during exacerbations. Analyses of the airway smooth muscle has shown a relationship between neutrophil infiltration, computed tomographic[20] measurements of air trapping and severity of airflow obstruction.[21] The investigators speculate that exposure to inflammatory mediators could affect the structure and contractility of the airway smooth muscle, contributing to peripheral airways obstruction.

Several reasons can explain the migration of neutrophils from general circulation to parenchyma lung in COPD. Firstly we know that in COPD patients, the intensity of the airway and blood neutrophilia, in both smokers and ex-smokers, correlates to the rate of decline in FEV_1, with the result that the severity of airflow limitation is related to airway inflammation.[26,27] So, the airways dysfunction is a key determinant of COPD severity and airway lumen neutrophilia is associated with this dysfunction.[18]

Furthermore, there is the increase of chemotactic protein in airways, in particular LTB4 and IL-8, which plays a fundamental role in co-ordinating and facilitating neutrophil migration by the activation of signalling pathways, cytoskeletal rearrangement, and changes in cell surface molecules.[28,29] Another important element is the micro-vascular alterations, caused by chronic inflammation, which are observed and appear to be clinically involved in COPD. In this regard, it has been well established that vascular endothelial growth factor (VEGF) plays an important role in the airway micro-vascular alterations in mild and moderate COPD disease.[30]

On the other hand, different mechanisms are involved in airway inflammations in sleep apnea patients. Indeed, a pro-inflammatory state has been reported at both systemic and local (upper airways) levels in these patients, and its impact remains to be elucidated. From a generic

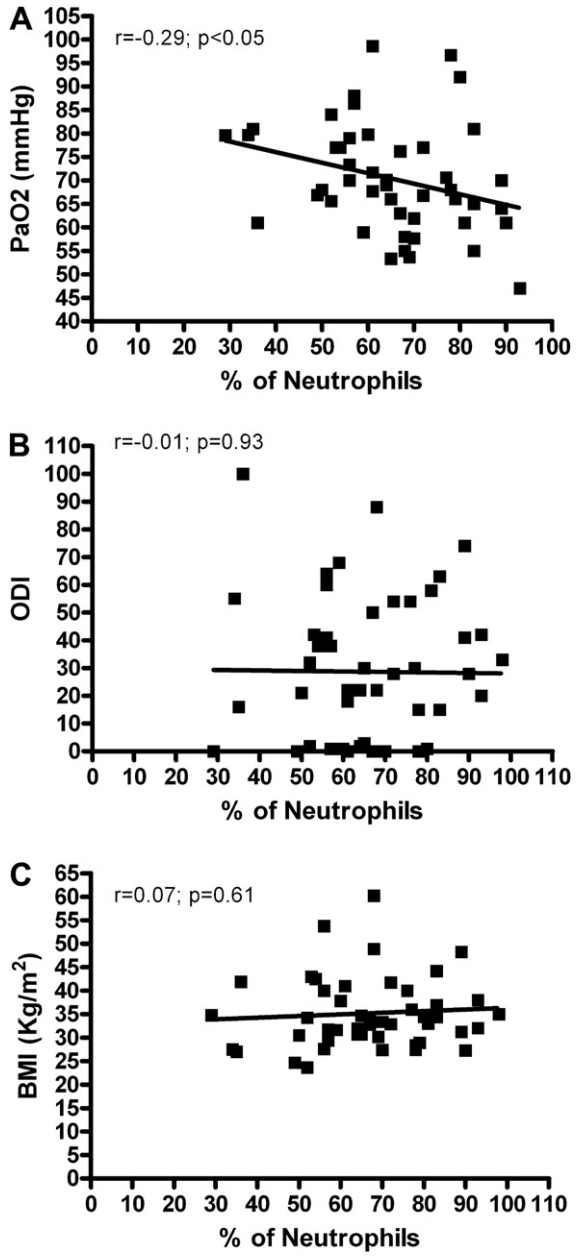

Figure 2 Correlation between neutrophils percentage and PaO_2 (A), ODI (B) and BMI (C) in OS, OSAS and COPD patients.

standpoint, many studies have demonstrated that patients with OSAS manifest high circulating levels of multiple biomarkers connected to both systemic and local inflammation.[31,32]

Several studies are available on hypoxia-mediated inflammation in airways of OSAS patients. In this regard, an increase in exhaled pentane and NO levels in OSAS patients and an increase in exhaled IL-6 concentrations were reported; indeed, in a previous study of our group, we described a higher percentage of neutrophils in the induced sputum of these subjects.[22,23]

The common hypothesis states that these phenomena are secondary to the upper airway mechanical injury and intermittent hypoxia, but further and more recent data indicate that their causes and consequences might be more complex than was originally thought. In fact, it has been demonstrated that apnea triggers a systemic inflammation by inducing changes in leukocyte function in an OSAS animal model,[33] whereas sleep deprivation per se induces spontaneous pro-inflammatory cytokine production by human monocytes.[34]

Besides that, many studies suggest that obesity alone is a direct cause of systemic inflammation[35] because adipose tissue produces different inflammatory adipokines such as leptin, and adiponectin in particular during conditions of hypoxia,[36] while the link between obesity and airway inflammation is not clear. Our data showed that there was no positive correlation between BMI and the increase of neutrophils percentage in airways, but we think that it is not sufficient to say that pulmonary inflammation and obesity are not linked. Thus, it remains unclear both whether obesity per se can induce pulmonary inflammation and what the link between this is.

Finally, our data show that only diurnal PaO_2 has a correlation with the airway percentage of neutrophils, but this is not true for noctural hypoxiemia because no correlation was found with TST90. We know that systemic hypoxemia contributes to TNF-α elevation in COPD[37] and also in OSAS, and that it is independent from obesity.[4] Thus, TNF-α and related cytokine levels could lead to the migrations of neutrophils into the airway, and this should be particularly true in patients with the Overlap Syndrome where, nocturnal tonic desaturations associated with intermitted episode of hypoxia, could have additional effects on an increased TNF-α level. However, in order to better understand the exact mechanisms and test the hypotheses, larger-scale studies are required.

There are several limits within this study, the most important being the lower number of enrolled patients as well as the difference in age between the control groups and patients. Thus, as stated above, more studies are needed to support our hypothesis.

In conclusion, these data show that in OSAS, COPD and Overlap Syndrome there is an increase of neutrophils in airway. May be neutrophils migration could be sustained by different mechanisms in these three diseases. However, Overlap Syndrome is the only disease in which upper and lower airway obstruction are both present and often associated with obesity. Indeed, it is for this reason that in this case each phenomena can likely contribute to sustained inflammatory status.

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Authors' contributions

Donato Lacedonia designed and co-ordinated the study, performed the statistical analysis and wrote the manuscript. *Francesco G Salerno* designed the study, recruited the patients and assisted in performing the statistical analysis, *Roberto Sabato* designed the study, recruited the patients and co-ordinated the study. *Palladino Grazia P* carried out the laboratory research and the patients' characterization for the classification of the different patient groups, *Maria Aliani* recruited the patients and helped with study design. *Giovanna E Carpagnano* participated in the interpretation of the results and critically reviewed the manuscript, *Maria P Foschino Barbaro* conceived and supervised the study as head of the lung research group, participated in its design and co-ordination and revised the manuscript.

Conflicts of interest

The authors have no conflict of interest to declare.

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